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PATENT APPLICATION
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Applicant: Jonathan S. Stamler
Serial No.: 08/616,371 Group Art Unit: 1811
Filed: March 15, 1996 Examiner: B. Celsa
For: METHODS FOR PRODUCING AND USING S-NITROSOHEMOGLOBINS

CERTIFICATE OF MAILING	
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231	
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DECLARATION OF JONATHAN S. STAMLER, M.D. UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Jonathan S. Stamler, M.D. of 101 Juniper Place, Chapel Hill, North Carolina 27514, hereby declare that:

1. I am the inventor of the subject matter described and claimed in Patent Application No. 08/616,371 entitled "Methods for Producing and Using S-Nitrosohemoglobins" filed on March 15, 1996.

2. I am named as a co-inventor on the published PCT application WO 93/09806, entitled "Nitrosylation of Protein SH Groups and Amino Acid Residues as a Therapeutic Modality," along with co-

inventors Joseph Loscalzo, Daniel Simon and David Singel. The Applicant listed for this PCT application is Brigham and Women's Hospital, and the international publication date is 27 May 1993. I have been provided with a copy of this published PCT application and I have reviewed its content prior to executing this Declaration.

3. WO 93/09806 describes certain research performed by the above-named co-inventors. I alone among the co-inventors performed the research described in Example 19 of WO 93/09806. At the time of the experiments discussed in this Declaration, I believed that I had made S-nitrosohemoglobin, but I learned later, with more experience in working with hemoglobin, that this had been a misinterpretation of the results, and that S-nitrosohemoglobin could not have been made as described in Example 19.

4. WO 93/09806 describes, in Example 19, pages 58-59, a procedure which was said to result in the formation of S-nitrosothiols in hemoglobin. The procedure is described incorrectly. The reagent stated as being used to cause S-nitrosylation of hemoglobin in Example 19 is missing entirely in the first paragraph at line 5. It might be inferred by the reader to be SNOAc (S-nitroso-N-acetylcysteine) because this is what appears on line 18 of page 58, but this is incorrect. According to my notebook records, (copies of which are provided as Exhibits A-C) these attempts to synthesize S-nitrosohemoglobin were actually done with acidified nitrite (NaNO_2 at pH 2, or in 1 N HCl), and not with equimolar SNOAc at pH 6.9 as the Examiner has inferred the reagent and the pH to be. Acidified nitrite cannot be used for synthesis of a functional hemoglobin (that is, hemoglobin capable of carrying oxygen); the predominant product is oxidized (methemoglobin) and the protein dissociates into non-functional subunits that cannot accurately be called hemoglobin.

The Saville assay was said to have been used to confirm the presence of S-nitrosothiols resulting from the procedure in the first paragraph of Example 19. However, no results of this assay are reported giving the number of S-nitrosothiols per molecule of hemoglobin. Not even a qualitative method confirms the presence of a SNO-hemoglobin product. Example 19 states, at page 58, lines 13-16, "Confirmatory evidence for S-nitrosothiol bond formation was obtained by spectrophotometry, demonstrated by the absorption maximum or 450 nm, as shown in Figure 28. This was demonstrated using NO⁺ equivalents in the form of SNOAc." The latter sentence does not follow from anything above it. As for the former sentence referring to Figure 28, it is reported (page 58, lines 13-15) that an absorption maximum of 450 was seen by spectrophotometry, demonstrating evidence of the S-nitrosothiol bond. One of skill in the art would know that the S-nitrosothiol bond has no characteristic maximum absorbance at 450 nm. Moreover, neither does S-nitrosohemoglobin. The maximum actually shown in Figure 28 is 540 nm. SNO-hemoglobin cannot be distinguished from hemoglobin by examining the absorbance at 540 nm or at 450 nm. A comparison of the spectra of the various forms of hemoglobin with Figure 28 allows one of skill in the art to conclude that Figure 28 shows a spectrum which does not resemble that of any form of hemoglobin. For comparison, see the spectra of deoxy-, oxy- and aquomet-hemoglobin reproduced from page 53 of *Allosteric Effects in Haemoglobin* by Kiyohiro Imai, Cambridge University Press, 1982, provided herewith as Exhibit D. What is shown in Figure 28 is not the spectrum of any form of hemoglobin. It is the spectrum of the azo dye whose absorbance is measured at 540 nm in the Saville assay. More, importantly, hemoglobin cannot be treated with 1 N HCl and still exist as the tetramer.

5. It is unclear from Example 19 what reagent was reacted with hemoglobin. However, I recently asked a person in my laboratory skilled in hemoglobin chemistry and assays to attempt to

synthesize SNO-hemoglobin according to the method one might assume is given, and the Examiner apparently has assumed is given, in the first paragraph of Example 19 of WO 93/09806. That is, $12.5 \mu\text{M}$ HbA₀ was incubated with $12.5 \mu\text{M}$ SNOAc at pH 6.9 for 5 or 20 minutes. The reactants were then separated, a step developed in our laboratory subsequent to those experiments described in Example 19, a step I know was not done in any experiment reported in Example 19. The Saville assay, as currently modified to measure S-nitrosohemoglobin (see below), was then used to measure any SNO-hemoglobin product in the high molecular weight fraction from the separation step. No SNO-hemoglobin was detected by the modified Saville assay. In fact, the values from the assay are in the negative range. The results of this recent experiment are shown in Exhibits E1-E3.

In fact, if SNOAc had been used as the reagent to attempt to nitrosylate hemoglobin, as was recently tested in our laboratory, it would have been impossible to assay for SNO-hemoglobin in any manner described in Example 19. No method described in the example includes a step to separate the nitrosylating reagent from whatever hemoglobin product might result, and the assay would detect SNO-groups on the SNOAc reagent as well as on any S-nitrosylated hemoglobin product that might be made. That is, in the Saville assay, there would be no way to tell whether the azo dye absorbing at 540 nm resulted from the displacement of NO⁺ from a product S-nitrosothiol or from the reagent SNOAc. In the experiment described in Example 19, I performed no separation step before attempting to assay for SNO-hemoglobin using the Saville assay, as shown by Exhibit B.

Further, it was not known at the time of the experiments in Example 19 that it is necessary to modify the Saville reaction to preserve the SNO-hemoglobin under assay conditions. If the standard Saville assay is used to assay SNO-hemoglobin, hemoglobin precipitates, confounding a background reading. Moreover, NO comes off the cysteine residues at pH 6.9, at which pH hemoglobin tends toward the T conformation.

The concentration of hemoglobin used in the procedure presents a further problem. It was reported as 12.5 μ M. Below hemoglobin concentrations of about 15 μ M, dissociation of hemoglobin into dimers is significant. See Perutz, M.F., pages 160-161 of "Molecular Anatomy, Physiology and Pathology of Hemoglobin" In *Molecular Basis of Blood Diseases* (G. Stamatyanopoulos et al., eds.), Saunders, Philadelphia, 1987, copy provided as Exhibit F. Preferential nitrosylation of dimers over tetramers would be a potential major confounding variable under these conditions.

6. WO 93/09806 reports, on page 58, lines 17-19, "As demonstrated by Figure 29, the UV spectrum of hemoglobin incubated with SNOAc shows no reaction at the redox metal (iron-binding site) of hemoglobin, over 15 minutes."

On the contrary, the original data do show a reaction at the heme iron (see Exhibit C). The skilled person would recognize that the spectra shown in Figure 29 are not characteristic of any single form of hemoglobin. It is unclear from Figure 29 and the description in Example 19 which form of hemoglobin -- oxy or deoxy, or a mixture of both -- was used as starting material, and what procedure was followed which resulted in each of the spectra shown. What one can observe in the spectra shown is a shift toward a UV maximum of 401 nm and a flattening of the peaks in the visible region, both characteristic of the spectrum of methemoglobin. (Refer to Exhibit D.) Thus, the reported conclusion of WO 93/09806 that the described method results in no reaction (that is, no oxidation) at the iron-binding site of hemoglobin is wrong.

The sentences purporting to describe the results shown in Figures 29 and 30 are subject to misinterpretation. They imply that S-nitrosohemoglobin was formed. However, there is nothing at all in Figure 29 or the other figures to indicate that S-nitrosothiol groups were formed on any kind of hemoglobin product. The conclusion that S-nitrosohemoglobin was formed

arose from a misinterpretation of the data. In subsequent experiments, it was learned that a pH of 6.9 cannot be used to form SNO-hemoglobin. At pH 6.9, hemoglobin tends toward the T conformation, and NO is unstable at the thiol of cysteine and will come off to oxidize the heme Fe. Maintaining the pH at 9 causes a different conformational state of hemoglobin (R rather than T)) and causes the thiol groups to become activated to become nitrosylated, as the pH is then higher than the pK.

7. Figure 30 does, as reported, show an absorption spectrum of NO bound to the hemes of deoxyhemoglobin, so called nitrosylhemoglobin. However, the stated method of treatment with acidified nitrite (NaNO_2 in 0.5 N HCl) does not produce nitrosylhemoglobin. Acidified nitrite cannot be used for synthesis of a functional hemoglobin of any sort; these severe conditions cause the formation of methemoglobin, predominantly, and the dissociation of the tetramer into monomer subunits that cannot function as hemoglobin and cannot be called hemoglobin.

A careful analysis of the data presented in Example 19 of WO 93/09806 leads one of skill in the art to the conclusion that there is no indication that any form of S-nitrosohemoglobin was ever made. Therefore, Example 19 of WO 93/09806 does not teach the production of S-nitrosohemoglobin, nor can it predict any property of S-nitrosohemoglobin.

8. The Examiner suggests (page 5, lines 10-13 of Office Action) that WO 93/09806 discloses that S-nitrosohemoglobin can be used in preparations "for the treatment of disorders by increasing oxygen capacity and transport." This has not been established. The binding reaction of O_2 to hemoglobin in the lungs goes to 100% (see Exhibit G, Figure 7-22, page 154 from L. Stryer, *Biochemistry* 3rd ed., W.H. Freeman and Company, New York, 1988). It is impossible to improve on this. Whether the affinity of hemoglobin for O_2 can be increased by nitrosothiols has not been

tested. WO 93/09806 presents no data to support this assertion by the Examiner.

Statements at page 58, line 26 to page 59, line 4 of WO 93/09806 draw an erroneous conclusion from a misinterpretation of data. The paragraph referred to reads as follows:

S-nitrosylation of hemoglobin does not result in the formation of methemoglobin and consequent impairment in hemoglobin-oxygen binding. Furthermore, an additional experiment demonstrated that S-nitrosylation of hemoglobin causes a leftward shift in the hemoglobin-oxygen association curve, indicating an increase in oxygen binding. Thus, the reaction between S-nitrosothiols and hemoglobin not only eliminates the inhibition of oxygen binding which occurs from the reaction with uncharged NO and generation of methemoglobin, but actually increases oxygen binding.

A "leftward shift in the hemoglobin-oxygen association curve" in this case resulted from the methemoglobin formed by the procedure which was intended to produce S-nitrosohemoglobin. (A hemoglobin-oxygen association curve is usually represented in a graph as the proportion of hemes in a hemoglobin preparation to which an O₂ ligand is bound, versus increasing O₂ pressure as in Figure 7-22 of Exhibit G.)

WO 93/09806 concludes that, "...the reaction between S-nitrosothiols and hemoglobin...actually increases oxygen binding." This is impossible, as binding of O₂ to normal human hemoglobin in the lungs is 100% efficient. WO 93/09806 teaches that an increase in O₂ affinity is desirable. On the contrary, an increase in O₂ affinity is characteristic of certain forms of hemoglobin in hemoglobinopathies. Such patients tend to suffer from polycythemia. See page 650 of *Harrison's Principles of Internal Medicine*, 14th ed., A.S. Fauci et al., eds., McGraw-Hill, 1998 (copy provided as Exhibit H).

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Jonathan S. Stamler, M.D.

Date